OBSERVATIONS ON STAINING THE FLAGELLA ON MOTILE BACTERIA.

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From a microscopical standpoint no branch of the investigation of micro-organisms is, perhaps, more interesting than the study of the flagella on motile bacteria. As their discovery has shown the structure of these organisms to be much more complex than was before supposed, the determination of the number and arrangement of these minute appendages with which the various species are provided is important, not only from the knowledge thus derived of their structure, but also as a possible aid in the differentiation of closely allied species.

The fact that certain motile bacteria were provided with flagella was made known early in the history of bacteriology, but there seems to have been no method devised until a comparatively recent time by which they could be carefully studied or by which their presence on other motile forms could be shown. The difficulty in detecting the flagella on bacteria in a fresh condition is well illustrated by the investigations of Dallinger and Drysdale, who saw only a few of these appendages in a preparation of *bacterium termo* after an incessant examination of nearly five hours. Although our instruments and methods have been much improved since that time, I believe that at present the satisfactory demonstration of flagella on living bacteria is one of the most difficult tasks known to microscopists.

It is through the development of the staining processes that the demonstration of these appendages on the great majority of motile bacteria has been made possible and, in many cases, comparatively simple. As early as 1877 Koch succeeded in staining the flagella on a certain number of the larger saprophytic bacteria. Since then other methods have been devised which are applicable to the smaller and also the pathogenic forms. By the aid of these methods the

flagella have become recognized as forming a part in the morphology of motile bacteria. This fact is important, as any difference found to be constant between the characters of the flagella of two bacilli will be as significant in differentiating them, the one from the other, as the presence or absence of spores or a difference in the form or size of the bodies of the germs themselves. It is for their differential value that a knowledge of the flagella may render valuable service to the practical bacteriologist.

In 1889 Loeffler published a method in which he introduced the principle of a mordant in staining the flagella and cilia on micro-organisms. By subjecting the preparations to the action of a mordant before they were brought into the staining fluid he succeeded in staining the flagella on a considerable number of bacteria. There were, however, many motile forms on which these appendages could not be detected. This fact led to further investigations, which resulted in the discovery by Loeffler of the principle "*that the alkali-producing organisms required an acid mordant, and that the acid-producing organisms required an alkaline mordant.*" This was based upon the results obtained by Petruschky,* who had found that a large number of bacteria would convert the reaction of a neutral medium (liquid) into either an acid or an alkaline one during their multiplication.

The importance of Loeffler's method in the acquisition of our knowledge of the flagella of bacteria has prompted me to present it here, in a form as condensed as possible, both for its own value and as a basis for my subsequent remarks: The formulæ for the preparation of the mordant and staining fluid, together with the details in their application, are as follows:

(1) *The Mordant.*—To 10 cc. of a 20 per cent. aqueous solution of tannin, 5 cc. of a cold saturated solution of the sulphate of iron, and 1 cc. of an aqueous or alcoholic solution of fuchsin, methyl-violet, or "*Wollschwarzlösung*" are added. The fuchsin is especially recommended.

The foregoing solution is to be regarded as the standard or stock solution to be used, and one which is successfully employed in staining the flagella of certain microbes, but for others the addition of an acid or alkali is necessary. Thus for the *comma bacillus* it is necessary to add to the 16 cc. of mordant ½ to 1 drop of a solution of sulphuric acid, equivalent to a 1 per cent. solution of sodium hydrate. For the *Typhoid bacillus* 1 cc. of a 1 per cent. solution of sodium hydrate must be added to the 16 cc. of mordant. By first determining whether the germ in question is an alkali- or acid-producing organism, the necessary quantity

* Centralblatt f. Bakteriologie u. Parasitenkunde, Bd. vi (1889), p. 625.

of the acid or alkaline solution to be added to the mordant can easily be determined by actual experiment.

(2) *The Staining Fluid.*—The staining fluid consists of a saturated solution of crystal fuchsin in the ordinary aniline water. As the aniline water is very nearly neutral, a saturated solution of fuchsin in it is sufficient. Better results may possibly be obtained by adding to this as much of a 1 to 1,000 solution of sodium hydrate, as it is necessary to bring it almost to a point of precipitation.

Cover-glass preparations should be made of the bacteria, to be studied in such a manner as to avoid all albuminous material. This is best accomplished by transferring a very small quantity of the growth from an agar or gelatine culture to a drop of sterile water on a cover-glass and thoroughly mixing; a small quantity of this is conveyed to a second cover-glass in a like manner; and again from the second a third preparation is made. By this treatment the albuminous substance is sufficiently diluted and the bacteria are isolated in an aqueous medium. The preparation is allowed to dry in the air. Sterilized hydrant water is preferred to distilled water for diluting the culture. It is of the utmost importance that the cover-glass should be free from all impurities. The film on the cover-glass is fixed by heat, but care must be taken not to overheat the preparation. The desired temperature can be obtained by holding the cover between the thumb and index finger over the flame, instead of passing it through the flame by means of forceps. By this method overheating is avoided. After heating, the film on the cover-glass is covered with the mordant and held over a flame until steam is given off. It is then removed, and after ½ to 1 minute the cover is rinsed in water, then in absolute alcohol, and again in water, until the mordant is completely removed. Care must be taken to remove all traces of the mordant from the cover-glass, as it would form, if present, a very troublesome precipitate with the staining fluid. The film is then covered with a few drops of the staining solution and the preparation again heated until the solution begins to vaporize. It is then removed from the flame, and after allowing the stain to act for about one minute, the cover is washed in a stream of water. The preparation can be examined immediately in water or allowed to dry and be mounted in balsam. The bacteria, with their flagella, should be deeply stained, resting upon a colorless background if they are distributed in a purely aqueous substance, but if albumen is present they are surrounded by a uniformly feebly stained medium, the intensity of which depends upon the quantity of albumen present.

By the use of this method the flagella have been stained on not only a large number of saprophytic, but also on all of the known motile pathogenic bacteria. Unfortunately the results usually obtained by this process are not satisfactory for the differential purposes suggested in a previous paragraph. The difficulty is not in simply demonstrating their presence, but in the inability to determine the number and arrangement of these appendages on the indi-

vidual bacteria. In all of the preparations that I have examined that had been stained by this method there were a large number of bacilli which exhibited no flagella, while on the others the number was variable; but lying between the bacilli were a greater or less number of flagella that had become detached from the bodies of the germs, presumably during the process of preparation. This is especially prominent in preparations of bacteria that are provided with a considerable number of these appendages, such, for example, as the hog cholera and typhoid bacilli. This fact renders it difficult, if not impossible, to determine the number of flagella with which the individual bacteria of a given species are provided.

Notwithstanding this difficulty which I have met, the method has given such satisfactory results in the hands of A. Messea, an Italian investigator, that he has proposed a systematic classification of bacteria based upon the number and arrangement of the flagella. This classification is as follows:

I. GYMNOBACTERIA.

II. TRICHOBACTERIA. { 1. *Monotricha.* 2. *Lophotricha.* 3. *Amphitricha.* 4. *Peritricha.*

The *monotricha* have one flagellum at one pole of the bacillus (*bacillus pyocyamus*). The *lophotricha* have a tuft or bunch of flagella at one pole of the bacillus (*bacillus of blue milk*). The *amphitricha* have a flagellum at each pole (*spirillum volutans*). The *peritricha* are provided with rows of flagella (*bacillus typhosus*).

Kruse,* in a review of Messea's article, says that this classification can have only a secondary value. It is evident that it would conflict very seriously with the natural grouping of the *schizomycetes,* as, for example, the *monotricha* would include bacilli, spirilla, and at least one micrococcus (the motile micrococcus described by Ali-Cohen).

In order to find some process by which I could determine more definitely the minuter details respecting the number, size, and arrangement of the flagella on especially the hog cholera and typhoid bacilli, I have made a considerable number of tests with Loeffler's and other methods† and with various modifications in both the preparation of

* Centralblatt f. Bakteriology u. Parasitenkunde, Bd. ix (1891), p. 107.

† Trenkmann's are the only methods, other than Loeffler's, that I have found to be of any special value. They involve, however, the same principles as those given by Loeffler, and consequently need not be discussed here. For reference and titles of the various articles on the demonstration of the flagella on motile bacteria see bibliography.

the solutions used and in the technique of their application. The result of this experimental work has been very largely negative, but the careful testing of each step in the various processes, especially that of Loeffler, has been productive in revealing a few facts which are deemed worthy of notice. Some of these have suggested certain slight modifications in the technique of Loeffler's process which promise to be of considerable value in the further study of these structures. As his method has already been quoted, I shall refer only to those sections of it for which modifications are suggested or which, for other reasons, are deemed worthy of special remark. These are as follows:

(1) *The Distribution of the Bacteria on the Cover-glass.*—It is of the utmost importance that the bacteria are properly isolated in the preparation. This can be accomplished very satisfactorily by the following process: The cover-glasses, after being *thoroughly* cleaned, are spread on a level tray. On each cover-glass is placed, by means of a flamed pipette, a moderately large drop of sterile water (distilled or hydrant). This will spread over the entire surface of the cover if it has been properly cleaned. The end of a flamed platinum wire is very gently touched to the surface growth of an agar or gelatine culture of the germ in question, after which it is very carefully immersed two or three times in as many places in the water on each cover-glass. A sufficient number of bacteria will adhere to the end of the wire to make from four to ten preparations. The cover-glasses are then placed in an incubator at a temperature of about 36° C., where they are allowed to remain until the water is evaporated.

Many of the bacteria by means of their power of locomotion will become separated from the clump of germs introduced by the wire, and will be found on the drying of the preparation to be distributed very satisfactorily around these centers. This natural separation of the bacteria prevents the breaking off of the flagella by stirring or other artificial means employed in securing the necessary distribution and isolation. In this manner I have isolated the bacteria in a preparation so that in many fields not more than a score of germs could be seen, and the excellect condition of their flagella warrant my recommending this method of preparation. The fact that the bacteria are properly isolated over only a small portion of the preparation is not necessarily an objection. Our hopes, however, are not yet fully realized, for there will generally be a few, often many, detached flagella lying between the bacteria, some of which are par-

tially or wholly deprived of their appendages. I have frequently observed clumps of flagella which gave the appearance of those belonging to an individual germ, the body of which had disappeared. As these were found in preparations made from old cultures, they suggest the possibility that the body of the germ is first to degenerate, or else that through some physiological process the flagella are detached from the bodies of the bacteria after a certain age is attained. These hypotheses are strengthened somewhat by the fact that in older cultures there appeared to be a greater number of detached flagella.

(2) *The Composition of the Mordant.*—The mordant recommended by Loeffler seems to be the most satisfactory of any thus far suggested for general use. I have found, however, that a mordant which contains only 10 per cent. tannic acid can occasionally be used with advantage. It can be more easily and thoroughly removed from the specimen, and consequently the formation of a troublesome precipitate with the staining fluid occurs less frequently. It was found that with the *hog cholera bacillus* it gave equally as good results as the one containing 20 per cent. tannin; with the *typhoid bacillus* it was not so satisfactory, and with a *water bacillus* a 20 per cent. tannin solution in the mordant was necessary to secure the staining of the flagella. From a limited number of experiments it seems quite probable that a variation in the quantity of the tannic acid in the mordant may be of much service in staining the flagella on certain bacteria where difficulties are experienced with the use of the mordant prepared after Loeffler's formula. Although I have tried a considerable number of the "fixing agents," I have thus far been unable to stain the flagella with the use of any mordant not containing tannic acid.

In applying the mordant I have met with better results by allowing it to act from two to three minutes. A convenient method of heating the mordant on the cover-glass is to pass it several times through the lower portion of the flame, which heats it sufficiently and prevents spattering. A safer method is to heat the preparation for the required time in a watch-glass containing the mordant.

The heating of the cover-class to fix the film is also an important condition in the success of the operation. Loeffler's method has the objection that it is difficult to heat the preparations uniformly. I have employed a hot-air chamber for this purpose, and, after a series of experiments varying in temperature from 90 to 180° C. and in duration from one-fourth to five minutes, have found that I

could obtain the best results by heating the preparations for one minute at a temperature of about 125° C. Little if any difference was noticed in preparations heated at a temperature varying from 120–140° C.

(3) *The Reaction of the Mordant.*—Experiments have shown that the statement made by Loeffler that an alkali-producing organism required an acid mordant and an acid-producing organism an alkaline mordant need not be taken in a very strict sense. This is illustrated with the hog cholera bacillus. As it is an alkali-producing germ, it would be necessary, according to Loeffler's statement, to add a certain quantity of the acid solution to the mordant in order to stain its flagella. Dr. Theobald Smith stained the flagella on this bacillus by the use of the neutral or standard mordant.* Further investigation has shown that its flagella can be stained by the use of the mordant containing a variable quantity of either the acid or sodium solution, good results being obtained when as much as 3 cc. of either solution was added to the 16 cc. of the mordant. It is better to add the sodium solution just before the mordant is to be used. I have also found that the flagella on the typhoid bacillus, an acid-poducing germ, can be stained by the use of either the acid or alkaline mordant. This deviation from Loeffler's results with the typhoid bacillus may possibly be due to the age of the germ, as the one I used had been preserved, by means of subcultures, for several years. The same range in the reaction of the mordant was found to be applicable to a few other bacteria.

(4) *The Staining Fluid.*—I have found that carbol fuchsin (10 cc. of a saturated alcoholic solution of fuchsin, 100 cc. of a 5 per cent. solution of carbolic acid) gives equally as good, if not better, results than the aniline water fuchsin recommended by Loeffler. It has the advantage that it can be kept for a much longer time, and consequently is ready for use at any moment. It has a less tendency to form a precipitate with any trace of the mordant that might be left on the cover. I have also stained the flagella very nicely with Loeffler's alkaline methylene blue.

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*Dr. Smith pointed out that the reaction of the culture liquid with some bacteria may be either acid or alkaline, according as glucose or other sugars are present or absent. These undergo fermentation with the formation of acids. In liquids free from sugars the reaction becomes speedily alkaline. Petruschky's classification of bacteria as acid- and alkali-producing is thus shown to depend largely on the composition of the culture medium.

(5) *The Age of the Culture to be Used.*—A careful examination of the growth from cultures of certain bacteria shows that the flagella can be stained on these germs from cultures varying in age from 20 hours to several weeks. For simply demonstrating their presence any aged culture, within the limits mentioned, can be used. In old cultures the flagella are more broken, and a larger number of detached appendages are observed. I have obtained my best results with a culture about two days old. The surface of agar seems preferable to gelatine for cultivating bacteria for this purpose.

In testing the various methods and their modifications I have stained the flagella on a considerable number of bacteria, among which I will mention a large motile bacillus quite common in Potomac water, the *bacillus fluorescens liquefaciens* and the *bacillus coli communis*. The last of these, like the *typhoid bacillus*, is provided with rings of flagella, and consequently belongs to the *peritricha*. The flagella on each of these species have been stained by the use of both an alkaline and an acid mordant.

Although a large amount of work has been done to develop satisfactory methods for staining the flagella on motile bacteria, there seem to be many conditions that are not yet fully understood. These must be carefully worked out by actual experiment before we will be able to determine accurately the specific character of the flagella on the different species of bacteria.

No flagella have been found on the swine plague and other non-motile bacteria, although a very large number of specimens have been stained by the same methods that I have successfully employed with the motile forms. This, together with the fact that with certain motile bacteria at least a few flagella can be seen in every stained preparation, eliminates from the writer's mind the doubt that has occasionally been expressed, that the long wavy or spiral filaments seen to radiate from the bacteria or, lying between them, do not belong to the germs with which they are associated.

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Bibliography.

1. *Cohn, F.* Untersuchungen über Bacterien. *Beiträge zur Biologie der Pflanzen*, Bd. I (1872), p. 126.

2. *Dallinger, W. H., and Drysdale, J. J.* On the existence of Flagella in Bacterium Termo. *The Monthly Microscopical Journal* (London), vol. xiv (1875), p. 105.

3. *Dallinger, W. H.* On the Measurement of the Diameter of the Flagella of Bacterium Termo. *Jour. of the Royal Mic. Society*, vol. i (1878), p. 169.

4. *Koch, Robert.* Untersuchungen über Bacterien. *Beiträge zur Biologie der Pflanzen*, Bd. II (1877), p. 416.

5. *Neuhauss, R.* Ueber die Geiseln an den Bacillen der Asiatischen Cholera. *Centralblatt f. Bakteriologie u. Parasitenkunde*, Bd. V (1889), p. 81.

6. *Loeffler, F.* Ein neue Methode zum Färben der Mikro-organismen im besonderen ihren Wimperhare und Geisseln. *Ibid.*, Bd. VI (1889), p. 209.

7. *Loeffler, F.* Weitere Untersuchungen über die Beizung und Färbung der Geisseln bei den Bakterien. *Ibid.*, Bd. VII (1890), p. 625.

8. *Trenkmann, Dr.* Die Färbung der Geisseln von Spirillen und Bacillen. *Ibid.*, Bd. VI (1889), p. 433.

9. *Trenkmann, Dr.* Die Färbung der Geisseln von Spirillen und Bacillen. *Ibid.*, Bd. VIII (1890), p. 385.

10. *Dowdeswell, G. F.* Note sur les Flagella der microbe der Choléra. *Annales de Micrographie*, T. II (1890), p. 367.

11. *Smith Theobald.* Einige Bemerkungen über Säure und Alkalibildung bei Bakterien. *Centralblatt f. Bakteriologie u. Parasitenkunde*, Bd. VIII (1890), p. 389.

12. *Moore, V. A.* A review of the methods of Demonstrating the Flagella on Motile Bacteria, with special reference to the Staining Processes. *American Monthly Microscopical Journal* (1891), p. 15.

13. *Messea, A.* Contribuzione allo studio delle Ciglia dei batterii e proposta di una classificazione. *Rivista d'igiene e sanita publica* No. 14, (1889), p. 513.